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Position-effect variegation and the new biology of heterochromatin

[Review article]
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Outline

Abstract

The phenomenon of position-effect variegation has long been used as evidence for the importance of chromosome position to gene expression in eukaryotes. Investigations published within the past few years demonstrate that position- effect variegation is caused by multiple mechanisms, and that direct tests of hypotheses are possible with numerous model systems.

Abbreviations

E(var) — *Enhancer of variegation* locus;
ORC — origin recognition complex;
PEV —position-effect variegation;
SIR —silent information regulator;
Su(var) — *Suppressor of variegation* locus.

Introduction

Heitz [1] recognized that some eukaryotic genomes are divided into two cytologically distinct entities, euchromatin and heterochromatin (Fig. 1). Euchromatin contains most of the single-copy DNA and mutable genes, decondenses during interphase, and replicates throughout S phase.

Heterochromatin defies simplistic definitions, but in general it contains few mutable genes, is rich in middle-repetitive and highly-repetitive sequences (including transposons), is constitutively condensed throughout the cell cycle, and replicates late in S phase (reviewed in [2]). Position-effect variegation (PEV) was first characterized by Muller [3] as the variable, but heritable, inhibition of euchromatic gene activity when artificially juxtaposed with heterochromatin by chromosome rearrangement (Fig. 1a). Numerous reviews published in the past few years have summarized the interesting history of researchers' accomplishments in this field [4][5][6][7][8][9•]. We now recognize that position effects include a broad array of phenomena, such as heterochromatin-induced inhibition of transcription and reduction in DNA copy number, telomere-induced position effects, interactions between genes on separate chromosomes (' *trans* -sensing effects'), and inhibition of heterochromatic genes and chromosome transmission functions (Fig. 1b). Thus, PEV provides a window for investigating the function and metabolism of heterochromatin, as well as other aspects of chromosome and nuclear organization. In this review, I will describe key developments in the field of PEV that have occurred during the past year, with special emphasis on new concepts resulting from molecular-genetic studies using tractable systems such as *Drosophila* and yeast. I will emphasize the viewpoint that chromatin assembly and transcriptional inactivation are not the sole mechanisms for PEV; there is mounting evidence that multiple PEV mechanisms exist, including nuclear compartmentalization and physical alterations of DNA.

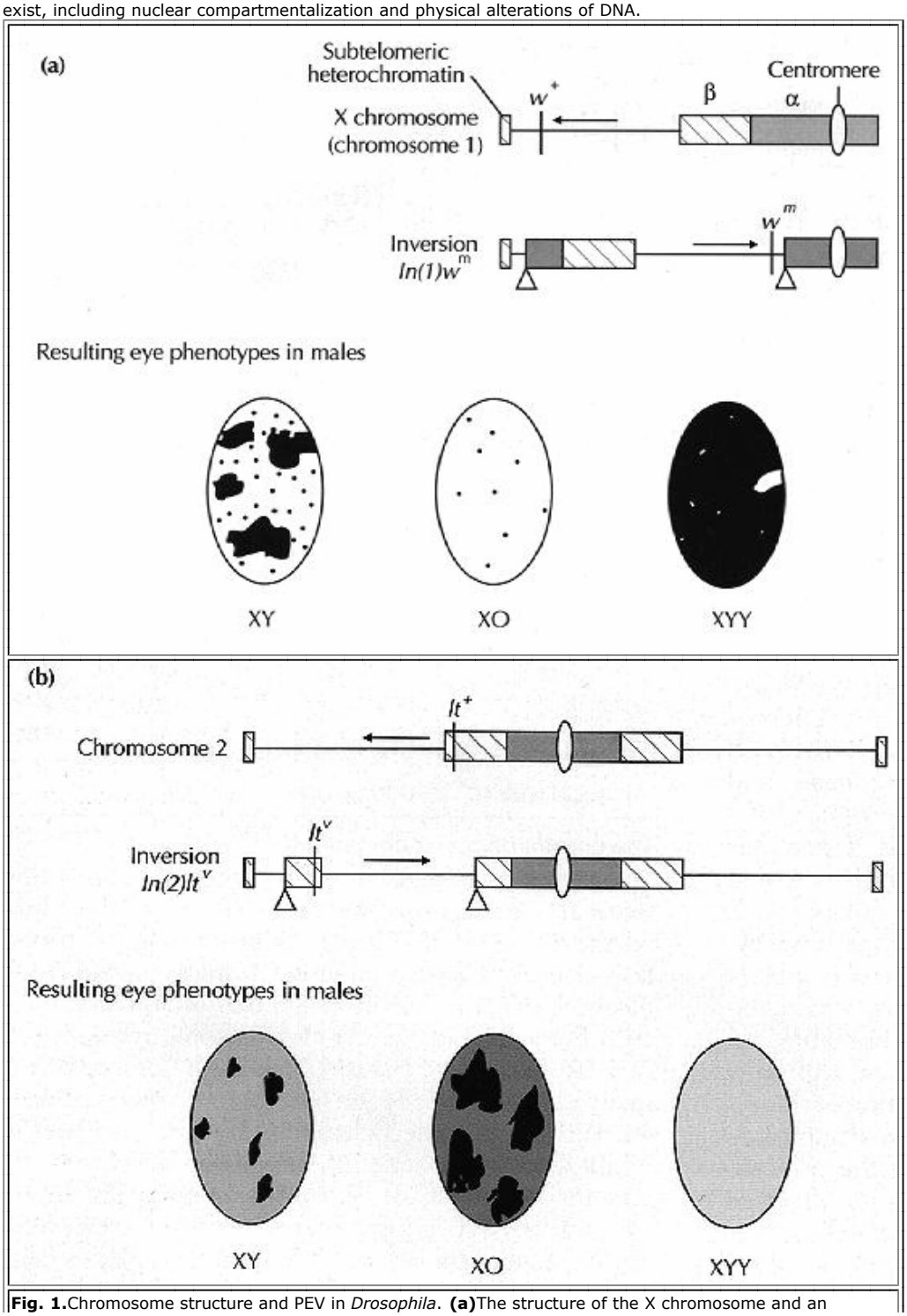


Fig. 1. Chromosome structure and PEV in *Drosophila*. **(a)**The structure of the X chromosome and an

inversion causing PEV. A chromosome rearrangement juxtaposing euchromatin and heterochromatin causes PEV (breakpoints are indicated by open triangles). This rearrangement brings the euchromatin (shown as a line), which includes genes such as the *white*(*w*) eye-colour gene, close to centric heterochromatin causing a *white* ^{mottled}(w^{m}) or variegated phenotype. The centric heterochromatin can be separated into α (gray) and β (diagonal bars) heterochromatin (reviewed in [75]). The α heterochromatin contains highly repeated satellite DNA, and some middle-repetitive elements, and is severely under- represented in dipteran polytene chromosomes. The β heterochromatin is the 'buffer' between euchromatin and α heterochromatin, contains middle-repetitive transposon-like sequences and some single copy genes, and is not under- represented in polytene nuclei. Subtelomeric heterochromatin resembles β heterochromatin in structure and can cause PEV of inserted genes [77•][79]. Eye phenotypes are shown for males with different sex chromosome constitutions (XY, XO, XYY). Dark areas indicate the normal red pigmentation (w^{+}expression) and white areas indicate ommatidia that lack w^{+}activity. Removal of the predominantly heterochromatic Y chromosome (XO males) significantly reduces total genomic heterochromatin and enhances the PEV in *trans*(more mutant), whereas additional heterochromatin (XYY males) suppresses PEV. **(b)**The structure of chromosome 2 and an inversion causing PEV. Normally heterochromatic genes, such as the *light*gene (*lt*), are moved into centromere–distal euchromatin [60]. In this case, the eye pigmentation phenotype responds to the amount of genomic heterochromatin in a 'reverse' manner to the heterochromatin-induced PEV in (a).

Return to text reference [1] [2] [3] [4] [5] [6]

Chromatin assembly and position-effect variegation in *Drosophila*

The chromatin assembly model

The hypothesis that chromatin compaction and transcr- iptional inactivation are the molecular mechanisms responsible for PEV arose from cytological observations on *Drosophila* polytene (endoreplicated) chromosomes (reviewed in [4]). Euchromatin juxtaposed with heterochromatin via chromosome rearrangement can display diffuse banding and high compaction normally characteristic of the heterochromatic chromocenter. This visible 'heterochromatinization' correlates with inhibition of gene function; those regions closest to the junction are most likely to appear compacted and to contain inactive genes. Elegant models (reviewed in [6]) have suggested that multimeric complexes of proteins normally present in heterochromatin are responsible for packaging large chromosomal domains in a repressed state. In these models, mass-action or self-assembly of the complexes is responsible for euchromatic 'spreading' of repressed gene activity, clonal inheritance of inactivation, and sensitivity of PEV to the dosage of heterochromatin and unlinked modifiers in the cell [10].

Although the chromatin assembly model has gained widespread acceptance, direct proof in multicellular eukaryotes, such as *Drosophila* , has been lacking (see below). In addition, new observations question the generality of previous cytogenetic characterizations of heterochromatinization. Directional spreading of chromatin assembly molecules is inconsistent with the discontinuous compaction seen upon close inspection of some variegating rearrangements [11]. Furthermore, recent studies have shown that two rearrangements present in the same cell can behave independently with respect to gene expression, compaction, and the binding of one heterochromatin-specific protein [12•][13•], discounting the hypothesis that cell-by-cell variations in gene expression are caused by differences in dosage of heterochromatinization proteins that are uniformly distributed in the nucleus. Recent findings support a role for other PEV mechanisms, such as nuclear positioning and somatic elimination, more consistent with these stochastic behaviors (see below).

Position-effect insulator elements and chromatin structure

Does chromatin structure actually change in response to PEV? Unfortunately, it has been difficult to analyze chromatin changes associated with PEV at the molecular or biochemical levels. The significance of chromatin structure in PEV has been questioned by a comprehensive analysis of the *white* gene in the inversion *In(1)w* ^{m4} . Only minor changes in DNase I sensitivity and nucleosome spacing were observed in response to this PEV rearrangement, despite extensive cytologically visible changes in polytene chromosome structure [14].

Putative chromosome domain boundary elements, such as specialized chromatin structures, can insulate chromosomally integrated genes from euchromatin-induced position effects [15] and block enhancer- mediated activation of transcription [16]. Recently, position- effect insulator elements have been identified from short DNA sequences associated with the nuclear scaffold [17], constitutive DNase I hypersensitivity [18••], and transcription factor binding [19][20•]. Surprisingly, insulator function appears to be highly conserved during evolution. A yeast scaffold attachment region element functions as an insulator in plant cells [17], and a chicken β -globin constitutive hypersensitive site insulates reporter gene function in human erythroid cells and *Drosophila* tissues [18••]. Further investigations will be required to determine whether primary sequence or secondary structure of these elements is recognized by other species, and how much of the protein machinery is conserved.

The identification of position-effect insulator elements suggests that chromatin accessibility can influence position effects. However, a role for insulator elements in heterochromatin-induced PEV is suggested by only one study [20•], which does not address the importance of chromatin assembly directly; for example, insulator elements could act by altering the nuclear compartmentalization of a gene through attachment to the nuclear membrane or scaffold. Chromatin changes, if demonstrated to occur, could be a secondary consequence of nuclear positioning.

Drosophila genes that modify position-effect variegation in *trans*

If the chromatin assembly model is correct, then loci encoding *trans* -acting modifiers of PEV should play a role in chromatin assembly, packaging, or maintenance. As many as 120 dominant suppressors (*Su(var)*) or enhancers (*E(var)*) of variegation exist in *Drosophila melanogaster* (see [6][8] for extensive reviews). One of the best studied modifier genes is the *Su(var)205* gene, which encodes the heterochromatin-binding protein HP1 [21]. The conservation of HP1 in evolutionarily distant species (mealybugs [22], *Drosophila virilis* [23], mice [24] and human [25•]), and the lethality of HP1 null alleles [26•], has suggested that HP1-like proteins are important for cell viability and/or development. However, the recessive lethal phenotypes of HP1 null animals are consistent with diverse roles for the protein, including activation of essential heterochromatic genes [27], chromosome transmission, or regulation of transposable elements [26•].

HP1 shares the 52 amino acid 'chromo domain' [28] or 'HP1/Pc box' [23] with the Polycomb protein (Pc), which regulates important developmental events by repressing euchromatic homeotic gene expression. A link between PEV and homeotic gene repression has been substantiated by the exciting demonstration that regulatory regions that respond to Pc group repression can induce variegation of an adjacent *white* gene [29••]. The fact that neither HP1 nor Pc proteins themselves bind DNA [30••], and the dose-dependent phenotypes associated with mutations at each locus [21][28], have led to the speculation that both proteins are involved in the assembly of different multimeric complexes that maintain repressed gene activity during development (reviewed in [31•]).

Does the chromo domain function to compact chromatin? Molecular dissection of HP1 protein functions indicates that nuclear localization and heterochromatin association functions map outside the chromo domain, but this study does not directly address the function of the chromo domain [32]. Monitoring the binding of mutant Pc proteins to euchromatic polytene chromosome sites has elegantly demonstrated that the chromo domain is necessary for proper Pc distribution [30••]. These results support the hypothesis that the chromo domain promotes protein–protein interactions, presumably between Pc, or HP1, and DNA-binding proteins that are responsible for localization of the complexes to specific chromosomal sites. As HP1 and Pc display distinctly different chromosomal distributions, their binding specificity must be encoded by minor differences between the chromo domains, or by another part of the amino termini. It is still unclear whether these complexes accomplish gene repression by higher-order chromatin compaction, nuclear compartmentalization, or other mechanisms (see below) [31•].

The involvement of proteins such as HP1 in PEV is likely to tell only part of the story. Only a few of the products from the hundreds of modifier loci have been analyzed by molecular cloning, and they vary in structure and potential function.
Cloned genes encoding modifiers include *Su(var)205* (HP1), *Su(var)(3)7* (function unknown, the protein contains unusually spaced zinc fingers [33]), *modulo* (a DNA-binding protein [34•]), and *Su(var)(3)6* (the protein phosphatase 1 catalytic subunit [35]). The identification of recessive PEV modifier mutations, whose phenotypes are not dose dependent [36], opens up the possibility that this new class of genes can be identified by direct genetic screening. The biological functions of even those genes that have been cloned are still obscure. A number of molecular mechanisms could be carried out by proteins associated with DNA, including chromatin compaction, but also nuclear localization, replication, nuclease activity, transposition and recombination. Our understanding of the diversity of mechanisms acting on heterochromatin will be greatly enhanced by direct demonstrations of the biological functions of the proteins. The isolation of readily clonable P-element induced *Su(var)* and *E(var)* alleles [37•][38] should greatly facilitate this important undertaking.

Telomeric and centromeric position effects in yeast

The relevance of *Drosophila* PEV models to other organisms has been validated by recent studies in yeasts (reviewed in [9•][39••][40•]). In *Saccharomyces cerevisiae* , inactive mating-type loci (*HML* and *HMR*) and genes inserted near telomeres display heritable, but reversible, repression reminiscent of the PEV phenotypes seen in multicellular eukaryotes [41]. As for PEV in *Drosophila* , telomeric silencing in yeast is directional and the extent of the silenced region can be modified by altering the dosage of the product of an unlinked locus (*SIR3* , a silent information regulator) [42•]. However, the yeast position effects include ~ 2–3 orders of magnitude less DNA, and inactivation of telomeric genes is normally less frequent, than heterochromatin-induced PEV in *Drosophila*.

The most direct biochemical and molecular evidence that PEV involves chromatin structure changes comes from detailed studies in *S. cerevisiae* and *Schizosaccharomyces pombe* . Resistance to methyltransferase activity [43][44], unusual nucleosome structures [45], suppression of silencing by histone H3 and H4 mutations [46], and hypoacetylation of histones [47] at telomeres and *HM* loci show that detectable chromatin changes are correlated with the silenced phenotype. *S. pombe* centromeric regions have been shown recently to induce PEV on inserted genes [48•], and the centromere central core is associated with unusual nucleosome spacing [48•][49].

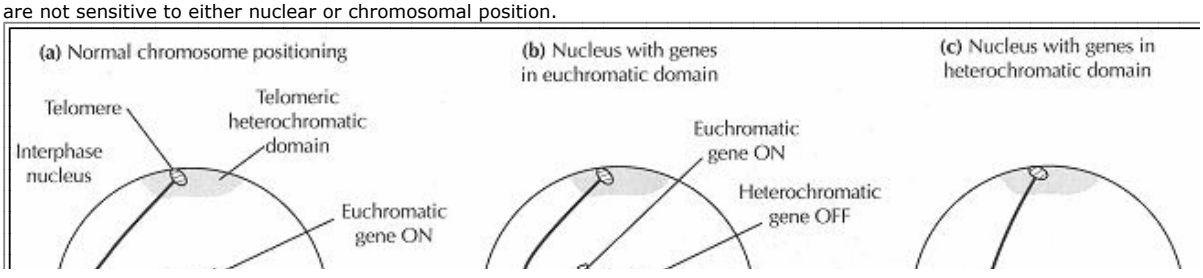
The primary cause of chromatin changes and PEV in yeast is still unknown, but progress in this area is rapid. The mapping of an origin of replication and a sequence required for silencing to the same 138 bp *HMR* element has forged a link between replication and silencing [50•]. The origin recognition complex (ORC) binds autonomously replicating sequences and is required for *in vivo* origin function. Elegant genetic [51••][52••] and biochemical [53••] analyses have demonstrated that the ORC is essential for silencing. ORC binding alone, rather than the initiation of replication, appears to be required to recruit proteins encoded by the *SIR* s to *HM* loci [54•]. It is still unclear whether ORC and subsequent SIR binding are required for establishment and/or maintenance of the repressed state [51••][52••][53••]. However, recent experiments investigating the cell-cycle dependence of activation of a telomere-silenced gene have suggested that replication may be necessary to reverse silencing (OM Aparicio, DE Gottschling, personal communication). The role of the RAP1 protein in PEV and localizing telomeres to the nuclear membrane will be discussed below.

Nuclear organization and position-effect variegation

Interphase nuclei display a characteristic organization (the Rabl orientation [55]). In general, centromeres and telomeres are clustered and are found associated with opposite poles of the nuclear envelope [56], their positioning and clustering being regulated during the cell cycle [57•][58•]. A number of studies published in the past few years have suggested that the positions of genes within the nucleus, not just within the euchromatic or heterochromatic regions of chromosomes, are important for normal expression (reviewed in [59•]). This constitutes a major change in our perception of the mechanisms responsible for PEV; genetic systems for investigating the functional significance of nuclear positioning are emerging rapidly.

It is difficult to explain the effects of distant structural changes on some PEV systems, and the discontinuous compaction of euchromatin associated with some rearrangements (see [12•] and text above), as the result of spreading chromatin compaction or decompaction along the chromosome. Tantalizing examples of unusual PEVs in *Drosophila* have suggested that heterochromatic compartment(s) exist within the nucleus and are important for normal gene function. The rare genes present in *Drosophila* heterochromatin display a 'reverse' position effect, that is, their function is inhibited when moved into euchromatin by chromosome rearrangement (Fig. 1b). PEV of heterochromatic genes, however, appears to be caused by increased distance from major heterochromatic blocks, rather than simple juxtaposition with euchromatin [60][61]. Interestingly, modifiers of PEV (such as changing the dosage of total genomic heterochromatin, or *trans* -acting genes) in general have opposite effects on PEV of euchromatic genes and PEV of heterochromatic genes [27] and chromosome transmission [62•] (Fig. 1b). Recent genetic studies have suggested that PEV of euchromatic genes is also influenced by nuclear position. Autosomal rearrangements that revert *brown* ^{Dominant} PEV move this locus (the normally euchromatic *brown* gene plus the adjacent insertion of a large block of heterochromatin) to the autosomal tips [63•]. Finally, PEV is enhanced by the removal of terminal sequences from a *Drosophila* minichromosome, even for terminal deficiency breakpoints up to 100 kb from the affected euchromatic gene [64][65].

These examples are consistent with models involving 'looping' of affected regions into heterochromatic (centromeric and/or telomeric) nuclear domains formed by Rabl configuration clustering. Looping into the heterochromatic domain would repress euchromatic gene function [63•], but would be essential for the function of heterochromatic genes (Fig. 2). Further investigations are required to test this hypothesis, such as cytological examination of the positions of ectopic heterochromatic genes in interphase nuclei [66] under variegating and non-variegating circumstances. It is worth noting that transformation experiments involving ribosomal genes demonstrate that their normal heterochromatic location is not required for RNA polymerase I transcription, nucleolus formation, or X–Y meiotic pairing [67][68], suggesting that these processes are not sensitive to either nuclear or chromosomal position.



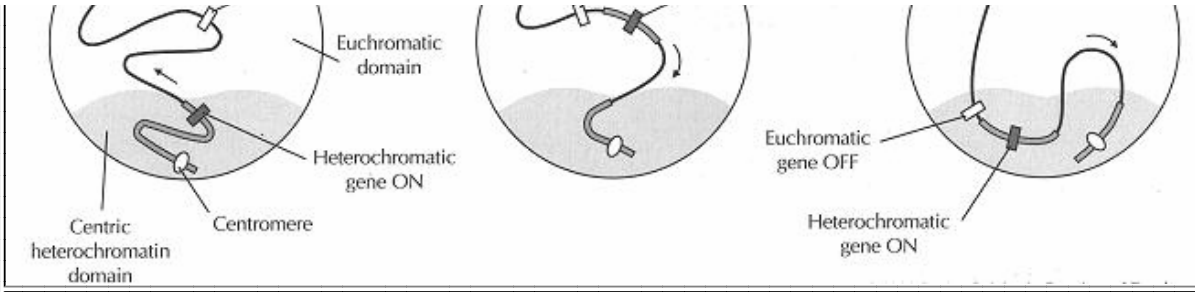


Fig. 2.A nuclear positioning model for PEV. **(a)**An interphase nucleus with heterochromatic domains formed by telomeric and centromeric clustering (Rabl configuration, see text). A representative chromosome is shown, with a heterochromatic gene indicated by a black box and a euchromatic gene indicated by an open box (see Fig. 1 for other symbols). **(b,c)**A rearrangement of the chromosome is shown, with the resulting gene expression being determined by the position of the genes within the nucleus. (b) Looping into the euchromatic domain would be essential for the expression of the euchromatic gene, but would repress the expression of the heterochromatic gene [60][61]. (c) Looping into the heterochromatic domain would be essential for the expression of the heterochromatic gene, but would repress euchromatic gene expression [63•]. Once located to the heterochromatic compartment, euchromatic genes could be inactivated by exclusion of transcriptional or replication machinery from the compartment, domain-induced changes in chromatin structure, or other types of modification (see text). In this model, the probability that a particular site would loop into a heterochromatic compartment would depend on the amount of heterochromatin at the site, the total amount of heterochromatin in the cell, and the distance between the site and the compartment.

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Results from studies of yeast position effects have suggested that telomere associations with the nuclear membrane are an important component of silencing and may be responsible for observed changes in chromatin structure. RAP1 protein binds to telomeric repeats [69], is responsible for telomere–telomere and telomere- -nuclear membrane associations (reviewed in [59•]) and is required for telomeric silencing [70][71•]. Furthermore, the SIR3 and SIR4 products are localized to the nuclear periphery, and mutations in either gene result in both loss of telomere–membrane associations and derepression of telomeric and *HM* silencing [72•]. The recent discovery that *RAP1* -dependent and *SIR2,3,4* -dependent silencing can be induced by C $_{1-3}$ A terminal repeats inserted up to 400 kb from the telomere argues for a critical role for the repeats, rather than telomere location *per se* (JB Stavenhhagen, VA Zakian, personal communication). The possibility that internal repeats accomplish silencing by looping to the telomere/nuclear membrane compartment has been suggested by the *RAP1* -dependence and the observation that C $_{1-3}$ A silencing is stronger at telomere–proximal locations. PEV associated with genes inserted at the *S. pombe* centromere requires a centromeric location, and not just central core sequences [48•]. Fission yeast centromeres are located at the nuclear periphery in interphase [49], but a functional link between the centromeric position effect and nuclear position has not been demonstrated. Perhaps the primary event in yeast silencing is localization to the nuclear membrane (dependent on *SIR3* , *4* / *RAP1* ?), followed by directional propagation of (*SIR1* , *2* ,/ *histone H4* / *ARD1* / *NAT1* -mediated ?) chromatin changes. Proof that telomere, *HM* or centromere associations with the membrane are sufficient for initiation will require the demonstration that returning genes to the periphery in *rap1, sir3* or *sir4* mutants restores silencing.

Position-effect variegation and physical alterations of heterochromatic DNA

DNA copy number reductions can be associated with position-effect variegation

The genetic and cytological characteristics of PEV are consistent with molecular mechanisms other than transcriptional inactivation via chromatin compaction and/or alterations in nuclear positioning. Physical modifications to the DNA, such as reduced gene copy number, could play a role in PEV, and provide an attractive explanation for the heritability of the repressed state through many cell divisions.

In dipteran polytene chromosomes, heterochromatic DNA copy number is reduced (under-represented) 64–1000-fold, with respect to the euchromatin [73]. Studies of the copy number of sequences in the *Drosophila* minichromosome *Dp1187* (Fig. 3) in polytene cells has provided strong support for the inclusion of adjacent euchromatin in heterochromatin-associated under-representation [74]. Although changes in euchromatic copy number were sufficient to account for phenotypic (*yellow* $^{+}$) variegation (Fig. 3), an additional effect on transcription could not be ruled out [74].

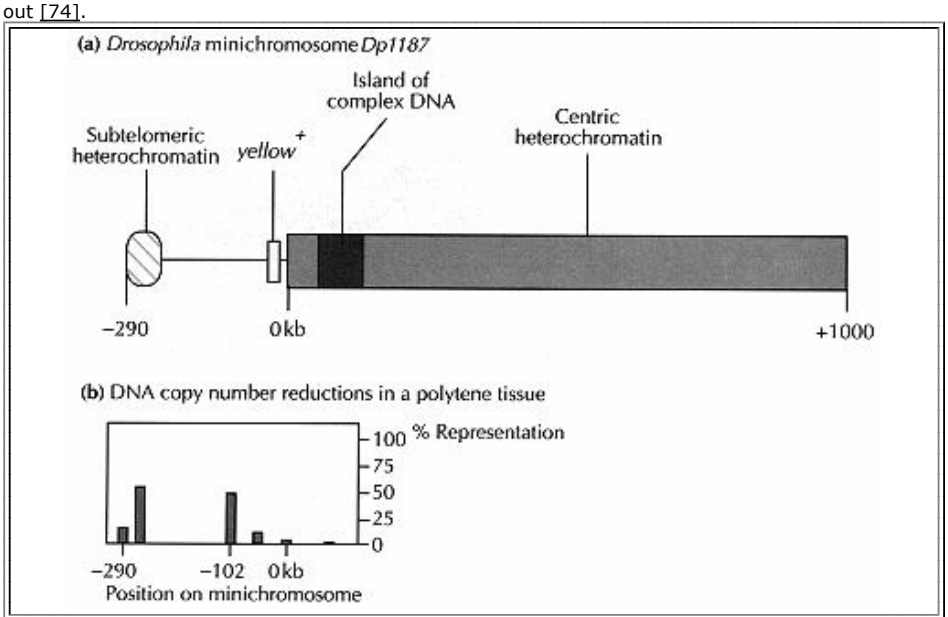

Fig. 3. Structure and under-representation of the *Drosophila* minichromosome *Dp1187*. **(a)** The molecular structure of the 1300 kb minichromosome *Dp1187*. The thin line represents euchromatin, which contains genes normally present on the tip of the X, including the *yellow* $^{+}$ body-colour gene located at - 20 kb. 0 kb marks the euchromatin–heterochromatin junction. The lightly tinted box represents the centric (α) heterochromatin, and the black box indicates the position of an 'island of complex DNA', which is a block of middle-repetitive and/or single copy sequences that is found between satellite-containing regions [74][77•]. Two other islands are present in the *Dp1187* heterochromatin (GH Karpen, unpublished data). **(b)** The DNA copy number for different regions of the minichromosome, relative to the same region on the normal X (% representation), is shown for XO salivary gland polytene nuclei [74][77•].

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The fact that some PEV rearrangements display euchromatic under-representation [74][76], whereas others do not [14][77•], suggests that heterochromatin is functionally diverse and that multiple mechanisms are responsible for PEV. The under-representation of juxtaposed euchromatin probably depends on the usual representation of heterochromatic regions present at the junction. For example, rearrangements involving β - heterochromatin (Fig. 1; reviewed in [75]) may not involve euchromatic under-representation, because at least some β - heterochromatin is known to be fully represented in polytene nuclei [78]. A striking example of the diversity of PEV mechanisms comes from a comparison of two different regions of the *Dp1187* minichromosome.

The strong inhibition of *rosy* $^{+}$ gene expression displayed by P-element constructs inserted in the subtelomeric heterochromatin (Fig. 3) involves only minor under-representation and is most probably caused by transcriptional inactivation [77•]. The subtelomeric insertions are located in a region rich in middle-repetitive elements, which is only 220 kb from the extensively under-represented *yellow* $^{+}$ gene adjacent to centric heterochromatin. Determining the roles of nuclear position and chromatin compaction in this and other telomeric position effects in *Drosophila* (see [77•][79] for references) requires further investigation.

Under-replication versus somatic elimination

What is the molecular mechanism responsible for heterochromatin- associated changes in DNA copy number? In *S. cerevisiae* , early replication origins become utilized late in S phase when inserted near telomeres [80•]. Under-representation in *Drosophila* could arise during polytenization because replication of heterochromatin is inhibited [81] or delayed [82•] (the 'under-replication' model), for example by packaging into a repressed chromatin structure, or inclusion in a nuclear compartment incompatible with normal replication. Alternatively, heterochromatic DNA may be physically removed during polytenization ('elimination'), and perhaps even in diploid somatic cells. DNA elimination has been documented in distantly related eukaryotic species (ciliates, nematodes, crustaceans, dipterans, and vertebrates), and frequently involves heterochromatin and polytenization (reviewed in [83][84••][85]).

Recent experiments utilizing the *Dp1187* minichromosome have suggested that elimination, rather than under-replication, is responsible for heterochromatic under-representation. DNA from a region of *Dp1187* displaying a gradient of under-representation in salivary gland nuclei (near 0 kb in Fig. 3) was analyzed by two- dimensional electrophoresis [86•]. Stalled replication forks, predicted by the under-replication model to be present in 33% of the molecules, were not found. Positive evidence for qualitative changes in chromosome structure associated with heterochromatin comes from recent pulsed-field analyses. Severely shortened chromosomes that lack the satellite DNAs normally present in *Dp1187* (Fig. 3) appear in polytenized tissues and are likely to retain only *Dp1187* euchromatin ([87•]; GH Karpen, AC Spradling, unpublished data).

Transposon-like elements (e.g. Tecs) are removed during macronuclear polytenization in *Euplotes* and *Oxytricha* [84••][88]. Perhaps transposon-like sequences in the 'islands of complex DNA' (Fig. 3) are responsible for under-representation in *Drosophila* . However, the elucidation of the exact mechanism of under-representation requires further experimentation, including in-depth structural analysis of developmentally altered molecules. The detailed restriction map of *Dp1187* heterochromatin (Fig. 3) and the isolation of a large number of minichromosome deletion derivatives (GH Karpen, unpublished data) will help in the testing of models by mapping *cis* sequences responsible for PEV and under-representation. Although exact mechanisms are not currently understood, the functional ramifications of heterochromatin undergoing developmentally regulated covalent changes in multicellular eukaryotes are numerous [4][85][87•], and warrant further exploration.

Conclusions and future prospects

The past year has seen renewed interest in PEV, yielding new information about PEV mechanisms and components, and producing manipulatable systems that hold promise for increasing our understanding in the immediate future. Major advances have come from detailed molecular-genetic analyses of telomeric and centromeric position effects and mating-type locus silencing in the yeasts *S. cerevisiae* and *S. pombe* . Further investigations should begin to elucidate the complex interaction between DNA replication and the establishment or maintenance of the silenced state, reveal chromatin structure and protein-component changes associated with position effects, and yield insight into the role of telomere associations with the nuclear envelope.

However, heterochromatin in multicellular eukaryotes, such as *Drosophila* and humans, is structurally and functionally more complex than that found in yeasts. It is encouraging that 'simplified' systems have been developed in recent years that allow heterochromatin and PEV to be studied with the specificity and directness necessary for progress. For example, correlating functions with the molecular structure of heterochromatin (e.g. *Dp1187*) will help in the elucidation of the nature of the *cis* sequences responsible for inducing PEV and the responsiveness of the variegating domains. From studies of *brown* and *light* variegation, and readily clonable modifier loci, we look forward to direct analyses of chromatin changes in affected tissues, molecular-cytological proof of the importance of nuclear positioning, and extensive analysis of the molecular and biochemical functions of modifier loci.

It is important to emphasize that direct proof of a primary role for chromatin assembly, the most widely accepted model for heterochromatin-induced PEV, is lacking in multicellular eukaryotes. Recent data, reviewed here, suggest that multiple mechanisms are responsible for the group of phenomena we call PEV, reflecting the structural and functional diversity of heterochromatin. It is likely that a number of the *Su(var)* and *E(var)* loci encode products that directly (e.g. DNA-binding proteins or packaging components, such as histones) or indirectly (e.g. regulatory phosphatases and kinases) influence chromatin structure. Investigators need to be more cautious, however, in assigning chromatin assembly or maintenance functions *ad hoc* to genes whose mutant products modify the phenotypes associated with PEV rearrangements. We cannot be blind to the exciting possibility that modifiers of PEV may function to regulate heterochromatic DNA elimination or inheritance functions, encode boundary functions that separate higher-order chromosome domains, regulate the position of domains within the nucleus, or control (as yet) undiscovered mechanisms responsible for the behavior and function of the mysterious entity known as heterochromatin.

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